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Use of a Recombinant Fluorescent Substrate with Cleavage Sites for All Botulinum Neurotoxins in High-Throughput Screening of Natural Product Extracts for Inhibitors of Serotypes A, B, and E▽†

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The seven serotypes of botulinum neurotoxin (BoNTs) are zinc metalloproteases that cleave and inactivate proteins critical for neurotransmission. The synaptosomal protein of 25 kDa (SNAP-25) is cleaved by BoNTs A, C, and E, while vesicle-associated membrane protein (VAMP) is the substrate for BoNTs B, D, F, and G. BoNTs not only are medically useful drugs but also are potential bioterrorist and biowarfare threat agents. Because BoNT protease activity is required for toxicity, inhibitors of that activity might be effective for antibotulinum therapy. To expedite inhibitor discovery, we constructed a hybrid gene encoding (from the N terminus to the C terminus, with respect to the expressed product) green fluorescent protein, then a SNAP-25 fragment encompassing residues Met-127 to Gly-206, and then VAMP residues Met-1 to Lys-94. Cysteine was added as the C terminus. The expressed product, which contained the protease cleavage sites for all seven botulinum serotypes, was purified and coupled covalently through the C-terminal sulfhydryl group to maleimide-activated 96-well plates. The substrate was readily cleaved by BoNTs A, B, D, E, and F. Using this assay and an automated 96-well pipettor, we screened 528 natural product extracts for inhibitors of BoNT A, B, and E protease activities. Serotype-specific inhibition was found in 30 extracts, while 5 others inhibited two serotypes.

Botulinum neurotoxins (BoNTs) are proteins produced by various strains of *Clostridium botulinum*, *Clostridium butyricum*, and *Clostridium baratii*. They are the most toxic substances known (4, 29, 39). There are seven BoNT serotypes, designated A through G, each expressed as a single-chain protein of 150 kDa. Subsequently, the last is cleaved by an endogenous bacterial protease(s) to yield the dichain molecule, consisting of a heavy chain (100 kDa) and a light chain (50 kDa), covalently linked by a disulfide bond. (An exception to this situation is BoNT E, which includes many strains wherein the toxin remains as the single-chain form.) The heavy chain of BoNT binds to receptors on peripheral cholinergic neurons, leading to internalization of the toxin, and the light chain is a zinc metalloprotease. Both chains must be present in the disulfide-linked holotoxin form to cause botulism; i.e., individual chains are not toxic (1, 15, 29, 39). Upon escape from endosomes into the neuronal cytosol, the light chain of each BoNT serotype cleaves only one peptide bond in its respective substrate. No two serotypes cleave the same bond. The synaptosomal protein of 25 kDa (SNAP-25) is the substrate for BoNTs A, C, and E. Serotype C also cleaves syntaxin. Vesicle-associated membrane protein (VAMP) is the target of BoNTs B, D, F, and G. Proteolysis at any one of these sites inactivates neurotransmitter exocytosis, leading to the flaccid paralysis of botulism (29, 39).

The stringent substrate requirement, cell specificity, and extreme efficiency of BoNT with respect to inhibition of neurotransmitter release have allowed clinicians to employ the toxin as a highly useful drug in an astonishing variety of human therapeutic applications where pathological conditions are caused by unregulated exocytosis of acetylcholine (9, 13, 40). In addition, recent results suggest that BoNT might be useful in cancer therapy, where its effects on tumor vascular structure enhanced the efficacy of radiation treatments and chemotherapy (3). Finally, BoNT heavy chain and genetically inactivated holotoxin have been studied as potential intracellular drug carriers targeted specifically to neurons (5, 11, 16, 43).

Unfortunately, BoNT is also a serious biowarfare and bioterrorism threat (4, 42). At present, the only treatment available for patients suffering from systemic botulism is supportive care through administration of antisera, to eliminate toxin still in the circulation, and mechanical ventilation. Recovery can take weeks or months. Once inside the neuronal cell, BoNT is invulnerable to antibodies, and there are no drugs available that can reach the toxin to reverse or mitigate its effects (4, 13, 19). Because the protease activity of BoNT is required for toxicity, efforts are under way in many laboratories to develop

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14. ABSTRACT

The seven serotypes of botulinum neurotoxin (BoNTs) are zinc metalloproteases that cleave and inactivate proteins critical for neurotransmission. Synaptosomal protein of 25 kDA (SNAP-25) is cleaved by BoNTs A, C, and E, while vesicle-associated membrane protein (VAMP) is the substrate for BoNTs B, D. F. and G. BoNTs are not only medically useful drugs, but are also potential bioterrorist and biowarfare threat agents. Because BoNT protease activity is required for toxicity, inhibitors of that activity might be effective for anti-botulinum therapy. To expedite inhibitor discovery, we constructed a hybrid gene encoding (from N-terminus to C-terminus, with respect to the expressed product) green fluorescent protein, then a SNAP-25 fragment encompassing residues met-127 to gly-206, followed by VAMP residues met-1 to lys-94. Cysteine was added as the C-terminus. The expressed product, which contained the protease cleavage sites for all seven botulinum serotypes, was purified and coupled covalently through the C-terminal sulfhydryl group to maleimide-activated 96-well plates. The substrate was readily cleaved by BoNTs A, B, D, E, and F. Using this assay and an automated 96-well pipettor, we screened 528 natural product extractsfor inhibitors of BoNTs A, B, and E protease activities. Serotype-specific inhibition was found in 30 extracts, while five others inhibited two serotype.

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specific inhibitors that might be useful as antibotulinum drugs or serve as model compounds for effective drug development (6–8, 22, 24, 30, 34, 35).

Progress in the identification of potential antibotulinum drugs has been aided by the availability of true high-throughput assays for BoNT protease activities. As noted above, BoNTs exhibit rigorous substrate requirements, and substrate modifications often lead to loss of functionality. Nonetheless, successful high-throughput assays, based on chemically modified synthetic peptides, have been devised for serotypes A, B, D, and F (2, 21, 32, 33). However, in all but one case, each substrate is suitable for only one serotype, with the exception being that for BoNTs D and F (32). Other types of assays for BoNT protease activities have been described (18, 20), but they are essentially enzyme-linked immunosorbent assays with many steps, and they require antibodies that can distinguish between cleaved and uncleaved forms of substrate.

In this report, we describe the design, production, and testing of a recombinant fluorescent substrate containing cleavage sites for all seven BoNT serotypes. The product is a simple protein that is easily purified in high yield. After covalent coupling to 96-well plates, the substrate was efficiently cleaved by BoNTs A, B, D, E, and F. The assay was readily adapted to an automated system and was used to screen an array of 528 natural product extracts for inhibitors of BoNTs A, B, and E.

MATERIALS AND METHODS

The BoNT substrate used in this work, GFPSV, consisted of (from N terminus to C terminus) green fluorescent protein (GFP), the linker sequence GSRG, SNAP-25 residues 127 to 206, VAMP residues 1 to 94, and finally, a cysteine residue as the C terminus. The supplemental material provides details of the cloning, expression, and purification of GFPSV.

For coupling to maleimide-activated 96-well plates (Pierce Chemical Co., Rockford, IL), GFPSV was equilibrated with 50 mM Tris–1 mM EDTA–0.1% Tween 20 (pH 7.5) by gel filtration on a column of Sephadex G-25 (GE Healthcare, Piscataway, NJ). Next, each plate well received 120 μl of 30 to 40 μg/ml of GFPSV. After 3 h at ambient temperature, the plates were kept at 4°C overnight. Wells were emptied and then incubated at ambient temperature for 60 min, first with 200 μl/well of 20 mM 2-mercaptoethanol in Tris–EDTA–Tween 20 (see above) and then with 200 μl/well of 10 mg/ml of bovine serum albumin in the same buffer. Finally, wells were washed three times with 200 μl of Tris–Tween 20 only (no EDTA). Wells were emptied and plates were stored at −20°C, where they were stable for at least 6 months.

Recombinant BoNT A and B light chains (A-Lc and B-Lc) were provided by Leonard Smith, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD (1, 15). Recombinant type E light chain (E-Lc) was purchased from Bishoushadhi Biotechnology (Dartmouth, MA). BoNT D and F holotoxins were obtained from Metabiologics, Inc. (Madison, WI). Myricetin, quercetin, caffeic acid, rutin hydrate, quercitrin hydrate, kaempferol, resveratrol, apigenin, piceatannol, and luteolin were purchased from Sigma Chemical Company (St. Louis, MO).

Six 96-well plates containing 528 natural product extracts were obtained under terms of an interagency agreement from the Natural Products Branch, Developmental Therapeutics Program, National Cancer Institute, Frederick MD. Three plates contained the dried residues of water-soluble material (aqueous extracts), while the other three contained a nearly identical set of extracts prepared with 1:1 methanol-methylene chloride (organic extracts). Each well contained approximately 0.5 mg of solids. Aqueous extract residues were dissolved in 100 μl of 40 mM HEPES–0.05% Tween 20 (pH 7.3), while those from organic extracts were dissolved in 100 μl of dimethyl sulfoxide.

An automated 96-well pipettor (Zymark, Hopkinton, MA) was used for liquid transfer and mixing steps in high-throughput assays. BoNT D and F holotoxins were preactivated as described previously (32). BoNT holotoxin and light-chain levels were adjusted to 1 μg/ml in HEPES-Tween buffer (see above). Ten microliters of natural product extracts was transferred into the corresponding wells of GFPSV-coated plates, followed by 90 μl of BoNT, and mixed. Control assay mixtures contained BoNT plus buffer, and blank assays contained buffer only. Fluorescence was read at timed intervals on a Wallac 1420 multiwell counter equipped with an automated plate stacker (Perkin-Elmer, Waltham, MA). Fluorescence is expressed in arbitrary units. All steps were done at ambient temperature.

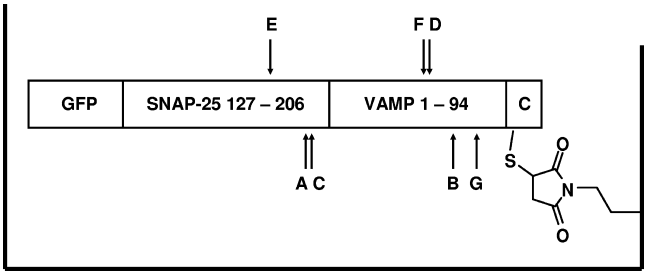

FIG. 1. A schematic representation of the recombinant fluorescent BoNT substrate GFPSV (not to scale) covalently bound through an *N*-ethylsuccinimide linkage to a plate well. Cleavage sites for the various BoNT serotypes are indicated by arrows.

High-pressure liquid chromatography (HPLC) assays of BoNT A-Lc and B-Lc protease activities were done with synthetic peptide substrates as reported previously (31, 38). The substrate for E-Lc was a peptide containing residues 167 to 199 of SNAP-25, and the assay buffer was 40 mM HEPES–0.05% Tween 20–1 mM dithiothreitol, pH 7.3. Other conditions were as referenced above for the other light chains. In HPLC assays to detect the presence of BoNT inhibitors in natural product extracts, the latter were adjusted to 5 mg/ml and diluted 1:10 into the assay mixtures.

Compounds from extracts were purified by reverse-phase HPLC on a 0.5- by 25-cm C_{18} column (Hi-Pore RP-318; Bio-Rad, Richmond, CA) using gradients of acetonitrile in 0.1% trifluoroacetic acid. Fractions were collected, lyophilized, redissolved in 40 mM HEPES–0.05% Tween 20 (pH 7.3) or dimethyl sulfoxide, and tested for BoNT protease activity inhibition as described above.

RESULTS

Figure 1 shows a schematic representation (not to scale) of GFPSV covalently bound to a plate well. In the SNAP-25 segment, BoNT E cleaves between R180 and I181, while BoNTs A and C hydrolyze adjacent bonds close to the C terminus (Q197-R198 and R198-A199, respectively). In the VAMP segment, BoNTs F and D cleave adjacent bonds (Q58-K59 and K59-L60, respectively), while the site for type B hydrolysis is the bond between Q76 and F77. Type G hydrolyzes the A81-A82 bond (28, 29, 40). Therefore, cleavage sites for all seven BoNT serotypes are present in the substrate with GFP on the N terminus, and proteolysis by any BoNT will release fluorescence into solution.

We tested the ability of BoNT serotypes A, B, and E light chains and of serotypes D and F holotoxins to cleave immobilized GFPSV (Fig. 2). Because covalently bound substrate was on the periphery of the fluorimeter light path, initial fluorescence was low, but it increased as the substrate was cleaved and the GFP-containing product diffused into solution. Figure 2A shows that immobilized GFPSV was hydrolyzed efficiently by A-Lc and B-Lc. Fluorescence reached maximum values in 30 to 40 min. At that point, adding fresh light chain to the wells had no effect on fluorescence values, indicating that the reaction had stopped because all available substrate had been cleaved and not because the light chain had become denatured (not shown).

Of equal importance is the observation that fluorescence in the blank wells (Fig. 2A, buffer only) did not change signifi-

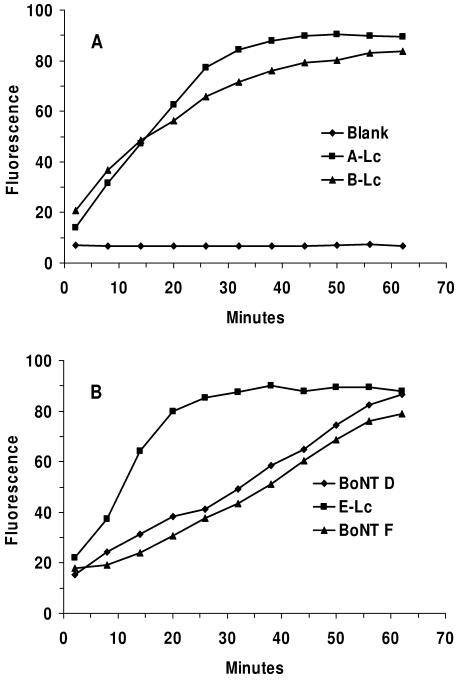

FIG. 2. Hydrolysis of the immobilized recombinant substrate by BoNT protease activities. Each point is the average from triplicate determinations, and in all cases, standard deviations were less than ±10%. (A) Blank (buffer only), A-Lc, and B-Lc. (B) BoNT D and F holotoxins and E-Lc.

cantly during the assay. Therefore, the substrate was tightly bound to the wells, and corrections for nonspecific release were not required.

Figure 2B depicts assay results for E-Lc and BoNT D and F holotoxins. The time course of fluorescence solubilized by E-Lc was similar to those for A-Lc and B-Lc. The holotoxins hydrolyzed GFPSV at lower rates than the recombinant light chains, but the concentrations of holotoxins (and therefore the light-chain components) were 7 nM, while those of recombinant light chains were 20 nM. Based on the semilinear portions of the curves, approximate net hydrolysis rates were 3.5 fluorescence units/min for 20 nM BoNT E light chain and 1.3 fluorescence units/min for 7 nM BoNT D holotoxin. Based on these findings, holotoxin at 20 nM would hydrolyze the substrate at about the same rate as 20 nM light chain. In summary, it was clear that the recombinant immobilized substrate was suitable for measuring the protease activities of BoNTs A, B, D, E, and F.

The release of fluorescence into solution was determined at timed intervals for all assays described in this report, but single-time-point assays could have been done also. In that case, an aliquot from each assay well is transferred into the corresponding well of a plain plate after 15 min of incubation, which stops the reaction immediately. Fluorescence of the transferred aliquot can be determined subsequently at any convenient time.

To test the utility of our immobilized GFPSV assay in BoNT protease inhibitor discovery, an array of 528 natural product extracts in six 96-well plates was obtained from the National Cancer Institute (NCI) repository. Plate wells contained dried

TABLE 1. Natural product plate numbers and sources of extracts

Plate no.	Source	Extraction solvent
96110120	Terrestrial plants	Water
96110125	Terrestrial plants	CH_3OH-CH_2Cl_2
12000707	Marine invertebrates	Water
11000831	Marine invertebrates	CH_3OH-CH_2Cl_2
99080203	Fungi	Water
99080111	Fungi	CH_3OH-CH_2Cl_2

residues from a collection of terrestrial plants, marine invertebrates, and various fungi. NCI plate numbers, sources of extracts, and extraction solvents are summarized in Table 1. The first well in each row was empty and was the control assay for that row. All plates were screened sequentially for inhibitors of BoNT A, B, and E protease activities by our high-throughput assay. Where present, the final concentration of dimethyl sulfoxide was 10%, but this had no effect on BoNT activities or on nonspecific release of immobilized substrate.

Representative results from high-throughput assays of BoNT A light chain are depicted in Fig. 3. Figure 3A shows selected wells from row B of plate 96110120 (aqueous extracts of terrestrial plants), while Fig. 3B displays results from row G of the same plate. In these examples, wells B3, B5, B9, G9, and G12 were selected for further analysis in HPLC-based inhibition assays.

In most cases, wells exhibiting significant inhibition of BoNT protease activities were easily discerned by visually inspecting assay plots. Nonetheless, to standardize the procedure, we determined hydrolysis rates for the linear portion of each curve and used these data to calculate percent inhibition values. An arbitrary cutoff of 50% inhibition was chosen; i.e., wells that

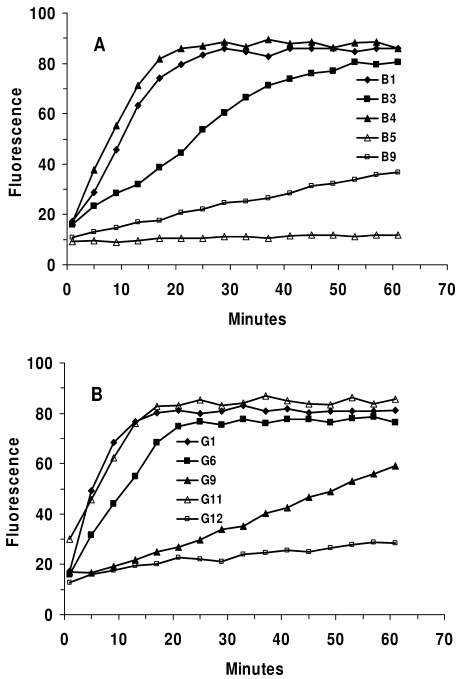

FIG. 3. Representative high-throughput assays of BoNT A protease activity in the presence of extracts from plate 96110120. (A) Selected wells from row B. (B) Selected wells from row G.

TABLE 2. Inhibition of BoNT A protease activity in HPLC assays by selected extracts from plate 96110120

Well no.	Hydrolysis rate (μmol/min/mg A-Lc)[a]	% Inhibition
Control	1.9 ± 0.023	0
B3	2.0 ± 0.034	0
B5	1.7 ± 0.011	11
B9	0.64 ± 0.018	66
G9	0.85 ± 0.033	55
G12	1.7 ± 0.014	11

[a] Initial hydrolysis rates were determined as described previously (31) and are the averages of triplicate determinations ± standard deviations.

inhibited ≥50% were selected for further analyses. From a total of 1,728 assays (96 wells × 6 plates × 3 serotypes), 170 wells met this criterion with respect to inhibition of BoNT A, B, and/or E protease activities. They were tested further in HPLC-based assays, and examples of the results are shown in Table 2. Although wells B3, B5, B9, G9, and G12 appeared to inhibit BoNT A-Lc in the initial high-throughput assays (Fig. 3), only B9 and G9 inhibited significantly when retested with the HPLC method.

An arbitrary cutoff of 30% inhibition was chosen for the HPLC assays, and wells that met this criterion are shown in Table 3, along with identifications of the organisms from which the samples were prepared. In most cases, inhibition was serotype specific. For example, well D8 of plate 12000707, an aqueous extract prepared from the green star coral *Pachyclavularia tosana*, inhibited BoNT A protease activity but not activity of BoNT B or E. Of the 35 extracts in Table 3, 6 inhibited BoNT A, 6 inhibited BoNT B, 18 inhibited BoNT E, and 5 inhibited BoNTs B and E.

Three extracts from the same plant, the African deciduous tree *Terminalia brownii*, were specific for BoNT A. The samples were in different wells because each had been prepared from a different part of the plant. To preserve the clarity of Table 3, this level of detail was not included for the other extracts but can be provided upon request.

TABLE 3. Extracts that inhibited at least 30% in HPLC assays

Serotype(s) inhibited	Plate no.	Well	Extract no.	Family	Genus	Species
A	12000707	D8	C19778	*Tubiporidae*	*Pachyclavularia*	*P. tosana*
	96110120	A7	N91270	*Combretaceae*	*Terminalia*	*T. brownii*[a]
	96110120	B9	N91304	*Combretaceae*	*Terminalia*	*T. brownii*[b]
	96110120	F11	N91346	*Combretaceae*	*Terminalia*	*T. brownii*[c]
	96110120	G9	N91314	*Euphorbiaceae*	*Synadenium*	*S. grantii*
	96110125	E12	N91371	*Geraniaceae*	*Geranium*	*G. aculeolatum*
B	12000707	A10	C19820	Unk[d]	Unk	Unk
	12000707	D9	C19798	*Raspailiidae*	*Echinodictyum*	*E. asperum*
	12000707	E11	C19842	Unk	Unk	Unk
	12000707	E8	C19782	*Polycitoridae*	*Eudistoma*	*E. factum*
	12000707	F2	C19696	*Xeniidae*	*Cespitularia*	*C. turgida*
	12000707	G6	C19748	*Didemnidae*	*Didemnum*	*D. molle*
B, E	12000707	G3	C19708	*Petrosiidae*	*Strongylophora*	*S. durissima*
	99080111	F5	F221165	Unk	Unk	Unk
	99080111	F9	F221267	Unk	Unk	Unk
	99080111	G2	F221017	Unk	Unk	Unk
	99080111	H4	F221153	Unk	Unk	Unk
E	12000707	A12	C19834	*Axinellidae*	*Axinella*	Unk
	12000707	A8	C19770	*Petrosiidae*	*Xestospongia*	Unk
	12000707	B12	C19848	*Polyclinidae*	*Aplidiopsis*	Unk
	12000707	B3	C19706	*Petrosiidae*	*Strongylophora*	Unk
	12000707	C2	C19682	*Petrosiidae*	*Strongylophora*	*S. durissima*
	12000707	C6	C19730	*Alcyoniidae*	*Sinularia*	*S. paralukari*
	12000707	D12	C19776	*Thorectidae*	*Cacospongia*	Unk
	12000707	E12	C19780	*Axinellidae*	*Dragmacidon*	*D. coccinia*
	12000707	E9	C19802	*Desmoxydae*	*Higginsia*	*H. bidentifera*
	12000707	F7	C19764	*Ancorinidae*	*Stelletta*	*S. splendens*
	12000707	H7	C19768	*Dasyaceae*	*Dasya*	*D. pedicellata*
	12000707	H9	C19816	*Geodiidae*	*Geodia*	*G. spheranthastra*
	96110120	A3	N91206	*Boraginaceae*	*Cordia*	*C. monoica*
	96110120	D8	N91292	*Celastraceae*	*Elaeodendron*	*E. buchananii*
	96110120	D11	N91342	*Fabaceae*	*Acacia*	*A. abysinnica*
	99080203	B4	F221166	Unk	Unk	Unk
	99080203	D3	F221154	Unk	Unk	Unk
	99080203	G8	F221268	Unk	Unk	Unk

[a] Leaf extract.
[b] Root bark extract.
[c] Stem bark extract.
[d] Unk, unknown.

Four of the five extracts that inhibited BoNTs B and E were methanol-methylene chloride extracts from fungi of unknown genus and species. Because of the enormous diversity of fungi and the inherent difficulties associated with classification, classification is not attempted for most of the unidentified fungal samples submitted to the NCI collection. Nonetheless, in many cases, classification can be accomplished for samples of interest.

The fifth extract that inhibited BoNTs B and E, well G3 of plate 2000707, was an aqueous extract of the sponge *Strongylophora durissima*. Apparently, the same extract in a different well (C2) inhibited only BoNT E. In fact, they were prepared from different samples of the same organism. The array of compounds present in an extract can be affected by many factors, such as the growth stage of the organism, collection site, and time of year. Therefore, the compound(s) responsible for BoNT B inhibition in well G3 might have been absent from C2, or inhibition could have been caused by entirely different compounds in the two cases.

Work is under way to purify and identify the inhibitory molecules in the extracts, as exemplified by Fig. 4. Figure 4A shows the fractionation of *Terminalia brownii* root bark aqueous extract (Table 3) by reverse-phase HPLC. Peaks 1 and 2 inhibited BoNT A protease activity, but the others did not. Figure 4B depicts the absorbance spectra of peaks 1 and 2. The peak 1 spectrum was relatively featureless, with significant absorbance only in the low-UV range. In contrast, the spectrum for peak 2 displayed maxima at 220, 256, 366, and 380 nm, which is characteristic of many plant polyphenols and flavonones. For comparison, the absorbance spectrum of myricetin (3,3′,4′,5,5′,7-hexahydroxyflavonone), which is found in many fruits and vegetables, is shown in Fig. 4C. This compound had absorbance maxima at nearly the same wavelengths as peak 2, although the relative values of the maxima differed from those of peak 2. Nonetheless, this finding suggests that the BoNT A inhibitor in peak 2 is a flavonone derivative.

Because of the results in Fig. 4, a collection of purified natural products, including flavonones and other types of compounds, were tested in HPLC assays for inhibition of BoNT A protease activity. The compounds were myricetin, quercetin, caffeic acid, rutin hydrate, quercitrin hydrate, kaempferol, resveratrol, apigenin, piceatannol, and luteolin. None inhibited at 50 μM in the assays (data not shown). These compounds have not yet been tested with other serotypes.

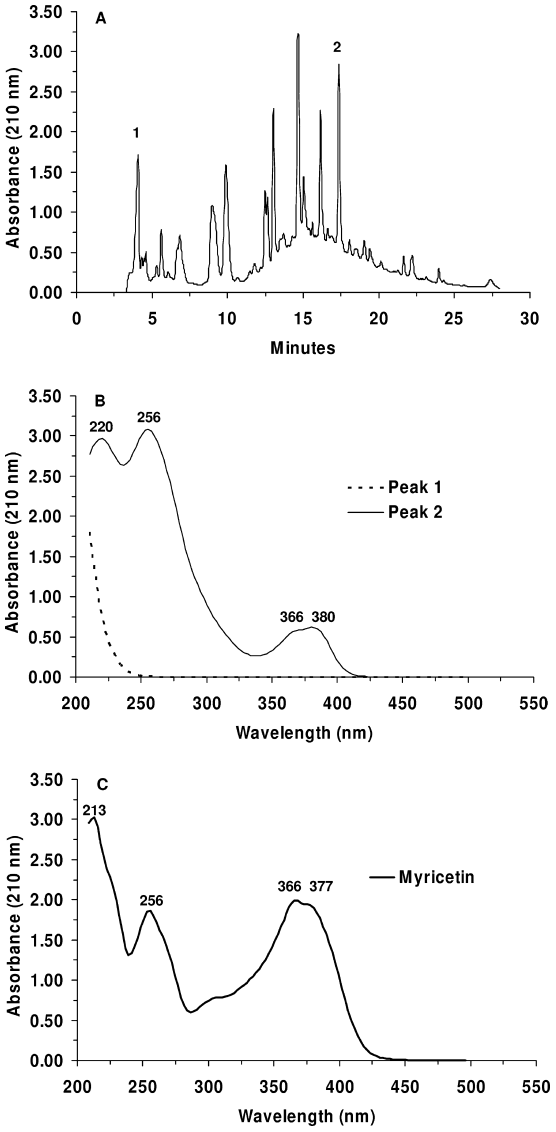

FIG. 4. Fractionation of *Terminalia brownii* root bark extract by reverse-phase HPLC. (A) Chromatogram of the column effluent monitored at 210 nm. Peaks 1 and 2 inhibited BoNT A protease activity. (B) Absorbance spectra of peaks 1 and 2. (C) Absorbance spectrum of myricetin.

DISCUSSION

The current lack of direct antibotulinum treatment modalities has generated an intense effort to discover drugs, based on inhibitors of BoNT protease activities, that can supplement or replace passive treatments and mechanical ventilation, which comprise the only currently available botulism therapies. To augment this research and accelerate the identification of promising BoNT inhibitors, there is a need for BoNT activity assays that are sensitive and economical and can be used with multiple BoNT serotypes. Previous high-throughput activity assays employed chemically synthesized, serotype-specific substrates that could be costly to produce. For example, Anne et al. (2) developed a fluorigenic assay for serotype B, but the substrate required de novo stereospecific synthesis of an intermediate that is not commercially available. Mangru et al. (21) developed an extremely sensitive microfluidic device to assay serotype A and B protease activities, but different chemically synthesized substrates were required for each serotype.

Our study addressed these problems by constructing a single recombinant polypeptide substrate, GFPSV, that included cleavage sites for all BoNT serotypes, a fluorescent protein for monitoring BoNT protease activity, and a cysteine residue to covalently bind the substrate to maleimide-activated 96-well plates. A typical growth and purification run yielded 30 mg of GFPSV, which is sufficient for 8,500 assays. The purified substrate and the prepared plates were stable and could be stored frozen for at least 6 months. Automation of the assay required only two liquid transfer steps and one mixing step. The protease activities of BoNT serotypes A, B, D, E, and F were measured in real time by monitoring the release of fluores-

cence into solution. Because the substrate was covalently bound to the wells, background fluorescence was low and did not change significantly during the assays. Therefore, the recombinant substrate and assay method described in this report constitute a highly effective and economical system to screen large collections of compounds for inhibitors of at least five of the seven BoNT serotypes. Indeed, the single-time-point assay method and a fully robotic environment would allow essentially continuous testing of arrays for BoNT inhibitors.

A major advantage of the recombinant substrate is the presence of potential toxin-binding regions distant from the sites of BoNT-catalyzed hydrolysis, regions not present in synthetic peptide-based substrates. On the other hand, internal folding or self-association of protein segments might occur in this relatively large substrate, diminishing the accessibility of such regions. However, in solution, SNAP-25 and VAMP exist mainly as random coils. During formation of the protein complex leading to acetylcholine release from neuronal cells, the first step is association of SNAP-25 and syntaxin, followed by binding of VAMP to form a highly structured ternary complex, containing extensive regions of alpha-helix, that is completely resistant to proteolysis by BoNTs. VAMP does not bind directly to either SNAP-25 or syntaxin alone (29). Therefore, while the possibility of self-association in the substrate cannot be excluded completely, the absence of binding between SNAP-25 and VAMP suggests that this is unlikely.

We tested the practicality of our assay by screening a collection of natural product extracts for BoNT protease inhibitors. We chose this matrix because natural products have been a source of medically useful compounds for many centuries. Almost 50% of currently prescribed drugs are natural products or derived from them (23, 26). Furthermore, recent reports have described antibotulism effects associated with natural products such as toosendanin, a triterpene derivative extracted from the fruit of the chinaberry, *Melia toosendan* (37), and with thearubigins extracted from black tea (27). However, in both cases, the mechanism of action involved inhibition of BoNT uptake into neuronal cells, and the compounds had no effect on BoNT protease activity. An extract prepared from green tea inhibited the zinc metalloprotease activity of anthrax lethal factor but not that of BoNT or tetanus neurotoxin (TeNT) (10). Caffeic acid, isolated from the shrub *Euonymus alatus*, was a potent inhibitor of matrix metalloproteinase 9 (25), but we report here that it did not inhibit BoNT A.

The first natural product proven unequivocally to inhibit BoNT protease activity was buforin I, an antimicrobial peptide purified from the stomach of the toad *Bufo bufo gargarizans*. This compound was a highly effective inhibitor of BoNT B, with a 50% inhibitory concentration of 1 μM (14). Michellamine B, a naphthylisoquinoline alkyloid isolated from the tropical vine *Ancistrocladus korupensis*, inhibited BoNT A but not BoNT B (6). An unidentified component of stinging nettle (*Urtica dioica*) was reported to inhibit BoNT A (17). Clearly, natural products are promising sources of BoNT protease inhibitors.

Screening 528 natural product extracts against three BoNT serotypes with the high-throughput assay described in this report revealed 170 samples that appeared to inhibit BoNT A, B, and/or E protease activity, but 135 did not inhibit significantly when evaluated further in HPLC-based assays. In general, a significant incidence of false positives is common to all fluorescence-based assays and not peculiar to our substrate or method of use. Many of the extracts were brightly colored, and the probable cause of apparent inhibition was the presence of compounds that quenched fluorescence. In contrast, HPLC-based assays yielded chromatograms that were inspected for the presence of the expected N-terminal and C-terminal hydrolysis products and for the remaining substrate, with appropriate retention times, and compared to standards. Calculations were based on peak areas and were not influenced by fluorescence-based artifacts (31, 38). Therefore, the 35 extracts that passed both primary and secondary screening procedures are likely to contain authentic BoNT protease inhibitors.

The inhibitory extracts exhibited clearly defined serotype specificities, as shown in Table 3. In addition to 30 monospecific inhibitors, five extracts inhibited BoNTs B and E but did not inhibit BoNT A. These findings demonstrate that the inhibitors are neither simple zinc chelators nor "promiscuous aggregating inhibitors" (36). The predominance of type E inhibitors is unexplained but is thought not to result from any inherent bias in the substrate or method, because for all tested serotypes, the assays were very similar with respect to hydrolysis rate, sensitivity, and stability of each BoNT protease activity during the procedure.

BoNTs C and G were not evaluated in this assay, but these serotypes have been shown to hydrolyze substrates (28, 41) that are present in GFPSV. Similarly, we did not determine if TeNT, another proteolytic clostridial neurotoxin closely related to BoNT, can cleave GFPSV. TeNT and BoNT B both hydrolyze the Q76/F77 bond in VAMP. TeNT can hydrolyze a synthetic peptide containing only residues 33 to 94 of VAMP (12), and our recombinant substrate includes the entire cytosolic portion of VAMP. Therefore, it is reasonable to predict that TeNT, BoNT C, and BoNT G will cleave GFPSV. In that case, our recombinant high-throughput assay would be suitable for the proteolytic activities of all eight clostridial neurotoxins.

BoNT C is the only clostridial neurotoxin that cleaves more than one neuronal protein, hydrolyzing syntaxin in addition to SNAP-25 (29, 39). Although the recombinant substrate does not include syntaxin, competitive inhibitors of BoNT C cleavage of SNAP-25 (i.e., those that compete with substrate for the enzyme active site) would protect against cleavage of syntaxin also, although to different extents, depending on the relative binding affinities of the two substrates for the toxin. Similarly, the assay will also identify inhibitors that bind to other locations on BoNT C, distant from the active site, that are common to the recognition of both substrates. Only those inhibitors that bind to sites on BoNT C unique for syntaxin recognition will not be detected by this assay.

In sum, the evaluation of 528 aqueous and organic extracts of plants, marine organisms, and fungi against the protease activities of BoNT A, B, and E light chains yielded serotype-specific inhibition in 35 samples, demonstrating that natural products are a highly productive source of BoNT inhibitors. The recombinant high-throughput multiserotype assay described here will enable an efficient and economical expansion of this effort. Finally, the utility of our substrate is not limited to screening natural product extracts. It can be used to test for inhibitors in any matrix that does not denature or otherwise prevent direct measurement of BoNT protease activities.

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